Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 23-29, 31, 33-39, 41, 43-49, 51, 53-61, 63, 65-73, 75, 77-81 and 83 are pending and under consideration in the application, with 23, 33, 43, 53, 65 and 77 being the independent claims in this group. Claims 30, 32, 40, 42, 50, 52, 62, 64, 74, 76, 82 and 84-96 were withdrawn from consideration. Claims 57 and 69 have been amended. Support for these amendments can be found in the specification, *inter alia*, at page 15, fourth full paragraph and page 23, second full paragraph. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicant(s) respectfully request(s) that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Objection to the Specification

The Examiner stated that the priority information on page 1 of the specification must be amended to indicate that Parent Application 08/852,824 is now U.S. Patent No. 6,060,272 and issued on May 9, 2000. Applicants have amended the specification accordingly.

The Examiner stated that on page 4 of the specification, Applicant has written "Brief Explanation of the Accompanying Drawings". Applicant respectfully disagrees. On page 5 the title "Brief Description of the Figures" is used to describe the figures.

The Examiner stated that the specification does not indicate the address of where clone 209004 was deposited. The specification has been amended to reflect the correct address for the ATCC and deposit information. Additionally, a statement concerning ATCC Deposit 209004 will be submitted upon receipt by the undersigned.

Accordingly, withdrawal of these objections are respectfully requested.

Rejections under 35 U.S.C. § 112, Second Paragraph

The Examiner rejected claims 57, 69, 77, 78-81, and 83 under 35 U.S.C. § 112, second paragraph, for allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. (Paper No. 8, page 4.)

The Examiner has stated that claim 77 "is indefinite because it is not clear which amino acids comprise the transmembrane domain of SEQ ID NO:4 so as to allow the metes and bounds of the claims to be determined." (Paper No. 8, page 4.) Claims 78-84 depend from claim 77. Applicants respectfully disagree with the Examiner's rejection.

The specification teaches that the transmembrane regions of G-protein coupled receptors, such as EDG-1-like protein are generally designated as TM1-TM7 and that each transmembrane region constitutes a stretch of 20-30 hydrophobic amino acids (page 2, line 32, page 3, lines 9-10). Since the amino acid sequence of EDG-1-like G protein coupled receptor is provided (SEQ ID NO:4), one skilled in the art can easily evaluate this sequence

and confirm that TM1-TM7 are, indeed, hydrophobic amino acid stretches. An amino acid sequence comparison of EDG-1-like G-protein coupled receptor (SEQ ID NO:4) and EDG-1 (SEQ ID NO:18), another G-protein coupled receptor, is also provided (*See* original specification, Figure 4). Watson *et al.* (cited by the Examiner) discloses the transmembrane regions of EDG-1. One of ordinary skill would understand which amino acids comprise the transmembrane regions of EDG-2.

As discussed at pages 2-3 of the specification, G-protein coupled receptors have a well-characterized and distinctive structural topology that is found in all members of the superfamily. In particular, several references report the manual and computer-aided alignment of hundreds of G-protein coupled receptors. *See, e.g.*, Probst *et al., DNA and Cell Biology 11*:1-20 (1992) (manual alignment of 74 unique human G-protein coupled receptors); and Oliveira, L. *et al., J. Computer-Aided Mol. Design 7*:649-658 (1993) (visual alignment of approximately 100 receptors and computer-aided alignment of 225 receptors). This structural homology further confirms the metes and bounds of the transmembrane regions of SEQ ID NO: 2. Therefore, claim 77 is definite, and Applicants respectfully request that the Examiner withdraw the rejection.

The Examiner states that claims 57 and 69 are indefinite "because it is unclear what 'activity' the G-protein coupled receptor has" and that "the 'activity' of the G-protein coupled receptor has not been disclosed in the claims nor the specification." (Paper No. 8, page 5.) Applicants have amended claims 57 and 69 by deleting the offending term "activity." Accordingly, the Examiner is respectfully requested to withdraw the rejection.

Claim Rejections under 35 U.S.C. § 101

Claims 23-29, 31, 33-39, 41, 43-49, 53-61, 63, 65-73, 75, 77-81 and 83 were rejected under 35 U.S.C. § 101 for allegedly not being supported by either a specific, substantial utility or a well established utility.

I. The Examiner Has Failed to Establish That An Artisan of Ordinary Skill Would Reasonably Doubt All Asserted Utilities.

Applicants note that the manner of making and using an invention disclosed in a specification must be accepted by the PTO "unless there is reason to doubt the objective truth of the statements contained therein." *In re Marzocchi*, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. 367, 369 (C.C.P.A. 1971); *see also Utility Examination Guidelines*, 66 Fed. Reg. 1092, 1098-99 (Jan. 5, 2001) ("*Utility Guidelines*"). Instances in which an assertion of specific utility is not credible are rare. *See* MPEP § 2107 (7th ed. Rev. 1, Feb. 2000). Indeed, the Federal Circuit recently affirmed the standard for making a utility rejection that was set forth in *In re Brana*, 51 F.3d 1560, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995):

> The PTO cannot make this type of rejection . . . unless it has reason to doubt the objective truth of the statements contained in the written description. *See Brana*, 51 F.3d at 1566, 34 USPQ2d at 1441.

In re Cortright, 49 U.S.P.Q.2d 1464, 1466 (Fed. Cir. 1999). The PTO's own guidelines provide:

> Any rejection based on lack of utility should include a detailed explanation why the claimed invention has no specific and substantial credible utility. Whenever possible, the examiner should provide documentary evidence . . .

> (e.g., scientific or technical journals, excerpts from treatises or books, or U.S. or foreign patents) to support the factual basis for the prima facie showing of no specific and substantial credible utility. If documentary evidence is not available, the examiner should specifically explain the scientific basis for his or her factual conclusions.

Utility Guidelines, 66 Fed. Reg. at 1098. Further, the Federal Circuit has recently articulated the standard for utility in light of *Brenner*:

> The threshold of utility is not high: An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. See *Brenner v. Manson*, 383 U.S. 519, 534 (1996); *Brooktree Corp. v. Advanced Micro Devices*, Inc., 977 F.2d 1555, 1571 (Fed. Cir. 1992) ("To violate § 101 the claimed device must be totally incapable of achieving a useful result"); *Fuller v. Berger*, 120 F. 274, 275 (7th Cir. 1903) (test for utility is whether invention "is capable of serving any beneficial end").

Juicy Whip, Inc. v. Orange Bang Inc., 185 F.3d 1364, 1366, 51 U.S.P.Q.2d 1700, 1702 (Fed. Cir. 1999).

The Examiner has not made the required showing that even one, much less all, of the disclosed utilities for the G-protein coupled receptor polynucleotides would be unbelievable in light of the teachings of the specification -- under either the standard set forth in *Juicy Whip* or the PTO's guidelines. The polynucleotides of the claims can be used for the diagnosis of cancer. (Original Specification, page 23, line 2.) For example, a polynucleotide sequence that is 90% identical to 30 contiguous nucleotides of SEQ ID NO:3 will hybridize to cells, tissues or classes of cells or tissues that express the EDG-1-like G protein coupled receptor. Therefore, the claimed polynucleotides certainly provide some identifiable benefit under *Juicy Whip*, and their utility is specific and substantial under the PTO's guidelines.

Thus, the Examiner has failed to provide any evidence or sound scientific reasoning to establish that an artisan would reasonably doubt all of the asserted utilities for the polynucleotides of the claims.

II. The Specification Discloses At Least One Specific Utility.

Applicants respectfully emphasize that the specification does disclose at least one specific activity of the EDG-1-like G-protein coupled receptor. Moreover, it is apparent that the instant case is not analogous to the situation in *Brenner v. Manson*, contrary to the Examiner's implication. (Paper No. 8, page 10.)

In *Brenner*, the issue was not whether a disclosed utility was sufficient. Rather, the applicant was trying to establish an earlier date of invention for the purpose of provoking an interference. 383 U.S. at 521. Indeed, the examiner's initial basis for refusing to declare an interference was that the applicant had *failed to disclose any utility* at all. *Id.* at 521. Thus, the issue in *Brenner* was whether the applicant had made an adequate "showing" to establish a prior date of invention, i.e., whether "the process claim has been reduced to production of a product shown to be useful" through actual demonstration of the utility. *Id.* at 534. The only evidence offered by the applicant to make this showing was a reference to an article by a third party showing the activity of an adjacent homologue of the subject steroid compound. *See id.* at 521-522. The appellate court agreed that the applicant had done nothing to show or demonstrate that the compound was indeed useful. *See id.* at 521. Thus, it upheld the rejection of the request for declaration of an interference. *Id.* at 536.

In contrast, the issue in the present case is whether the instant application

explicitly teaches at least one utility that meets the requirements of § 101. Applicants submit that the specification discloses a number of specific uses for EDG-1-like G-protein coupled receptor molecules. The Examiner stated that "there is no evidence of record or any line of reasoning that would support a conclusion that the claimed receptor of the instant application was...useful for diagnosis, prevention, and treatment of disease, such as cancers" and that "neither the specification nor the art of record disclose any disorders that can be effected by interfering with the activity using the EBI-2 receptor or fragments thereof." (Paper No. 8, page 10, lines 22-24 and page 12, lines 4-6.) However, the specification states that the antagonists of the G-protein coupled receptor may be used to treat, *inter alia*, cancer. (*See* original specification, page 23, line 2.) Additionally, the polynucleotides of the invention may be used for the detection of cancer.

The specification also states that examples of inhibitors of the EDG-1-like G-protein coupled receptor include antibodies, small molecules, and soluble forms of the receptor. (Original specification, page 23, second full paragraph; page 24, first full paragraph and second full paragraph). The use of these EDG-1-like G-protein coupled receptor molecules to treat, for example, cancer is a specific use that is not generally applicable to all G-protein coupled receptors, much less to all proteins. *(See, e.g., Revised Interim Utility Guidelines Training Materials* ("*Utility Training Materials*") example 4, pages 32-33 (the use of an uncharacterized protein as an amino acid source or a protein supplement are uses that apply to "virtually every member of a general class of materials such as proteins" and therefore are not specific utilities under the facts of example 4.)). Thus, Applicants submit that the specification discloses at least one specific utility for the EDG-1-like G-protein coupled receptor.

III. At Least One Asserted, Specific Utility Is Substantial.

The Examiner stated that "neither the specification nor the art of record disclose any activities or properties that would constitute 'real world' context for use for the claimed EDG-1 receptor and fragments thereof." (Paper No. 8, page 13.) Applicants respectfully disagree.

Applicants respectfully emphasize that the specification discloses at least one specific and substantial utility for the EDG-1-like G-protein coupled receptor. A substantial utility is one that defines a "real world" use. (*Utility Training Materials* at page 6.) The use of EDG-1-like G-protein coupled receptor molecules to treat and/or diagnose, for example, cancer, is a substantial utility as it provides a benefit to the public. Thus, at least one asserted use for the EDG-1-like G-protein coupled receptor is specific and substantial, as well as credible, as discussed further below.

Real-world value of an invention requires that "one skilled in the art can use a claimed discovery in a manner which provides some immediate benefit to the public." *Nelson v. Bowler*, 626 F.2d 853, 856, 206 USPQ 881, 883 (CCPA 1980). Furthermore, "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a specific utility" (MPEP page 2107, column 2, lines 16-20). The specification and the claims provide support for the use of the EDG-1-like polynucleotides as a diagnostic for detecting the expression of EDG-1-like GPCR (Specification page 27, third full paragraph). Indeed, the use of EDG-1-like polynucleotides as a diagnostic for cancer provides a public benefit and has real world value. *In re Rinehart* states:

If the record as a whole would make it more likely

> than not that the asserted utility for the claimed invention would be considered credible by a person of ordinary skill in the art, the Office cannot maintain the rejection. 531 F-2d 1048, 1052, 189 USPQ 143, 147 (CCPA 1976)

Applicants assert that the claimed invention is supported by a specific and substantial utility.

IV. At Least One Asserted, Specific And Substantial Utility Is Credible.

Although the Examiner has failed to carry the burden of showing that the disclosed utilities are unbelievable, Applicants submit herewith documentary evidence that the utility of the EDG-1-like G-protein coupled receptor in cancer has been demonstrated in the art.

The Federal Circuit has set forth the standard by which an asserted utility is established through supporting data. The Federal Circuit pointed out that its "predecessor court has noted that adequate proof of any pharmacological activity constitutes a showing of practical utility." *Cross v. Izuka,* 753 F.2d 1040, 224 U.S.P.Q. 739 (Fed. Cir. 1985), *citing Nelson v. Bowler,* 626 F.2d 853, 206 USPQ 881 (C.C.P.A. 1980) and *Rey-Bellet v. Englehardt* 493 F.2d 1380, 181 U.S.P.Q. 453 (C.C.P.A. 1974). Specifically, the Federal Circuit held:

> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of a pharmacological activity is reasonably based upon the probative evidence.

Cross v. Izuka, 753 F.3d at 1050.

Furthermore, utility can exist for therapeutic inventions "despite the fact that an applicant is at a very early stage in the development of a pharmaceutical product or

therapeutic regimen based on a claimed pharmacological or bioactive compound or composition." MPEP § 2107 (III) at 2100-27. "Usefulness in patent law . . . necessarily includes the expectation of further research and development. The stage at which an invention in this field becomes useful is well before it is ready to be administered to humans." *In re Brana*, 51 F.3d at 1568.

There is clearly a direct nexus between the G-protein coupled receptor (EDG-1-like) and cancer. The Examiner's attention is respectfully drawn to a post-filing date publication by Van Brocklyn *et al.* that reports the cloning of EDG-6, a G-protein coupled receptor which has an amino acid sequence that is nearly identical to EDG-1-like protein (there are only four amino acid differences at positions 78, 90, 122, and 142). (*See* Van Brocklyn, J. *et al.*, *Immunobiology 95(8)*:2624-9 (2000), attached herewith.) Van Brocklyn implicates the G-protein coupled receptor (EDG-6) in mitogen-activated protein kinase (MAPK) signal transduction pathway. Moreover, the authors of this publication indicate that SPP mediated EDG-6/EDG-1-like signaling may protect human T-lymphoblastoma cells from Fas or ceramide-induced apoptosis. Thus, this publication by others confirms the nexus between the G-protein coupled receptor (EDG-1-like protein) and cancer.

The disclosure in Van Brocklyn *et al.* confirms the credibility of using EDG-1-like G-protein coupled receptor polynucleotides to detect EDG-1-like expression in cancer. (*See* original specification, paragraph spanning pages 22-23; page 15, fourth full paragraph). Therefore, the documentary evidence cited above confirms the credibility, as well as the specificity and substantiality, of at least one asserted utility for the EDG-1-like G-protein coupled receptor molecules of the present application.

In view of the facts set out above, Applicants submit that a skilled artisan would not reasonably doubt that the claimed polynucleotides can be useful in making EDG-1-like proteins, generating antibodies, or diagnosing and/or treating cancer. As such, Applicants assert that the presently claimed invention possesses a specific, substantial, and credible utility that constitutes a patentable utility under 35 U.S.C. § 101. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 101 be reconsidered and withdrawn.

Claim Rejections under 35 U.S.C. § 112, First Paragraph

Claims 23-29, 31, 33-39, 41, 43-49, 51, 53-61, 63, 65-73, 75, 77-81 and 83 were rejected under 35 U.S.C. § 112, first paragraph for alleged lack of enablement. (Paper No. 8.)

For the reasons discussed above in response to the rejection under 35 U.S.C. § 101, the claimed invention is supported by a specific, substantial and credible asserted utility. The Examiner "should not impose a 35 U.S.C. 112, first paragraph, rejection grounded on a 'lack of utility' basis unless a 35 U.S.C. 101 rejection is proper." M.P.E.P. § 2107 (IV) at 2100-28. Therefore, because the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejections under 35 U.S.C. § 112, first paragraph, based on the alleged lack of utility of the claimed invention, should be withdrawn.

Claims 23-29, 31, 33-39, 41, 43-49, 51, 53-61, 63, 65-73, 75, 77-81 and 83 were rejected under 35 U.S.C. § 112, first paragraph for alleged lack of written description. (Paper No. 8.) Applicants respectfully traverse.

The written description requirement serves to ensure that the inventor had possession,

as of the filing date, of the claimed subject matter. However, "how the specification accomplishes this is not material." *In re Wertheim*, 191 U.S.P.Q. 90, 96 (C.C.P.A. 1976). In *Regents of the University of California v. Eli Lilly & Co.*, the court stated, "[a] description of a genus of cDNAs may be achieved by means of [1] a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus ***or*** [2] a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus." *Regents of the University of California v. Eli Lilly & Co.*, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997) (emphasis added), *cert. denied*, 66 U.S.L.W. 3688 (1998). Thus, the Federal Circuit has indicated that the written description requirement for generic claims directed to genetic material may be satisfied by providing the sequences of a representative number of members which fall within the scope of the genus ***or*** by providing a recitation of common structural features of the members of the genus.

Additionally, according to the court in *Eli Lilly & Co.*:

> In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can *distinguish* such a formula from others and can *identify* many of the species that the claims encompass. *Accordingly, such a formula is normally an adequate description of the claimed genus.*

Eli Lilly & Co. at 1406 (emphasis added).

Applicants submit that the present claims recite generic formulae that indicate with specificity the subject matter that the claims encompass. One of ordinary skill in the art can *distinguish* the present generic polynucleotides from others and can *identify* many of the species encompassed by the present claims. Accordingly, the present claim recitations are "*adequate description[s] of the claimed genus.*" *Id.* (emphasis added).

Additionally, for the reasons stated below, Applicants assert that the reference polynucleotide and polypeptide are representative of the claimed genus in satisfaction of the first test set forth in *Eli Lilly & Co.* Applicants also assert that the recitation of the complete sequence of the reference polynucleotide and encoded polypeptide constitutes a recitation of the structural features common to the members of the genus, in satisfaction of the second test. Concerning the first test, Applicants assert that the reference nucleic acid sequence (e.g., independent claims 23, 53 and 65) which falls within the scope of its genus, is representative of the genuses of polynucleotides encompassed by the claims. For example, polynucleotides at least 90% identical to the specific polynucleotide sequence will show activity, such as hybridization activity, much like the specific polynucleotide itself. Thus, polynucleotides comprising the specific sequences are exemplary of the structure of the variants within the genuses.

Concerning the second test, Applicants assert that the recitation of the *complete* EDG-1-like G-protein coupled receptor polynucleotide sequence is a recitation of the structural features common to the members of its respective genus because the polynucleotides in the genus have at least 90% of their nucleic acid sequence (i.e., primary structure) in common with the reference polynucleotide. Likewise, the recitation of the *complete* sequence of the EDG-1-like G-protein coupled receptor polypeptide is a recitation of the structural features common to the members of its respective genus because the polynucleotides in the genus encode polypeptides having at least 90% of their amino acid sequence (i.e., primary structure) in common with the reference polypeptide.

The claims are also adequately described under the PTO's guidelines and *Synopsis of Application of Written Description Guidelines*. According to the guidelines:

> [t]he written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by . . . disclosure of relevant, identifying characteristics, *i.e.*, [1] structure or other physical and/or chemical properties, [2] by functional characteristics . . . ***or*** [3] by a combination of such identifying characteristics.

Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112, ¶1, *"Written Description" Requirement*, 66 Fed. Reg. 1104, 1106 (Jan. 5, 2001) ("*Written Description Guidelines*") (emphasis added).

Thus, the guidelines indicate that a representative species may be adequately described through its structure, through its functional characteristics, ***or*** through a combination of its structure and function.

As discussed above, each member of each genus is described by reference to its sequence, i.e., its structure. The members of claim 23, for example, each shares at least 90% of its polynucleotide residues with 30 contiguous nucleotides of SEQ ID NO:1. Indeed, because of the degeneracy of the genetic code, a number of the polynucleotides within the scope of claim 23 encode a polypeptide that shares 100% of its amino acid residues with the reference polypeptide. Each member of the claimed genuses shares substantial sequence identity with the relevant reference sequence. Thus, there is *not* substantial variation within each genus. The reference polynucleotide species is, therefore, representative of the respective genus and the description of the complete sequence for the representative species is an adequate written description for the genuses encompassed by the claims.

Once the skilled artisan had the DNA and amino acid sequence of a given G-protein coupled receptor, the artisan could easily locate the extracellular, intracellular and transmembrane domains for that G-protein coupled receptor and identify many functionally

important amino acid residues. In addition, the specification specifically discloses assays that can be utilized for identifying and isolating polynucleotides that encode these polypeptides. (See specification, page 11, lines 16-30 and page 15, lines 18-26.) Accordingly, Applicants submit that one skilled in the art would not have to engage in an undue amount of experimentation to make and use the claimed invention.

Further, one of ordinary skill in the art can readily envisage polynucleotides comprising SEQ ID NO:3 because such sequences can be combined with sequences known in the art such as vectors, regulatory regions, or marker sequences. (*See, e.g.,* original specification, paragraph spanning pages 15-16). Any substantial variability within the genuses would therefore arise due to elements that are not part of the inventors' contribution. Additionally, procedures for making variants having at least 90% identity were conventional in the art at the time of filing. Although there may be a degree of variability among the 90% identical variant species to which the claims are directed, the necessary common structural features remain, e.g., SEQ ID NO:3. Therefore, one skilled in the art would recognize that Applicants were in possession of the claimed genus.

For all of the above reasons, Applicants respectfully assert that the Examiner has failed to meet the required burden in presenting evidence or reasons why those skilled in the art would not recognize the claimed invention from the disclosure. Moreover, the specification conveys with reasonable clarity that Applicants were in possession of the claimed invention. Thus, Applicants submit that the pending claims fully meet the written description requirements of 35 U.S.C. § 112, first paragraph under both tests set out in *Eli Lilly & Co.* and under the PTO's *Written Description Guidelines* and *Synopsis*. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, are

respectfully requested.

Rejections Under 35 U.S.C. § 112, First Paragraph - Deposit Rules

Claims 23-29, 31, 33-39, 41, 43-49, 51, 53-61, 63, 65-73, 75, 77-81 and 83 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly being nonenabled for reciting deposited biological material. The Examiner required that the specification be amended to recite the address of the ATCC, and required that particular averments be made concerning the deposit.

Applicants have amended the specification accordingly. As amended, the specification discloses the address of the depository. "Statement Concerning the Deposited cDNA Clone" which contains the necessary averments will be submitted upon receipt by the undersigned. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicant(s) therefore respectfully request(s) that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicant(s) believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the

number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Version with markings to show changes made

In the Specification:

The sentence beginning at page 1, line 1:

This Application is a continuation of U.S. Application No. 08/852,824 filed May 7, 1997, now U.S. Patent No. 6,060,272, issued May 9, 2000, the disclosure of which is incorporated herein by reference in its entirety.

The paragraph beginning on page 7, line 17:

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of Figures 3A and 3B (SEQ ID NO:4) or for the mature polypeptide encoded by the cDNA of the clone deposited with the ATCC, 10801 University Boulevard, Manassas, VA 20110-2209, as ATCC Deposit No. 209004 on 4/28/97.

The paragraph beginning on page 10, line 21:

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide

sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also code for a proprotein which is the mature protein plus additional 5[1]' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

In the Claims:

57. (Once amended) The polynucleotide of claim 53, wherein said nucleic acid encodes a polypeptide which [as G protein-coupled receptor activity.] <u>binds an antibody having specificity for the polypeptide of SEQ ID NO:4.</u>

69. (Once amended) The polynucleotide of claim 65, wherein said nucleic acid encodes a polypeptide which [has G protein-coupled receptor activity.] <u>binds an antibody having specificity for the polypeptide of SEQ ID NO:4.</u>

Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6

James R. Van Brocklyn, Markus H. Gräler, Günter Bernhardt, John P. Hobson, Martin Lipp, and Sarah Spiegel

EDG-6 is a recently cloned member of the endothelial differentiation gene (EDG) G protein-coupled receptor family that is expressed in lymphoid and hematopoietic tissue and in the lung. Homology of EDG-6 to the known sphingosine-1-phosphate (SPP) receptors EDG-1, EDG-3, and EDG-5 and lysophosphatidic acid (LPA) receptors EDG-2 and EDG-4 suggested that its ligand may be a lysophospholipid or lysosphingolipid. We examined the binding of [^{32}P]SPP to HEK293 cells, transiently transfected with cDNA encoding EDG-6. Binding of [^{32}P]SPP was saturable, demonstrating high affinity (K_D = 63 nmol/L). Binding was also specific for SPP, as only unlabeled SPP and sphinganine-1-phosphate, which lacks the trans double bond at the 4 position, potently displaced radiolabeled SPP. LPA did not compete for binding of SPP at any concentration tested, whereas sphingosylphosphorylcholine competed for binding to EDG-6, but only at very high concentrations. In addition, SPP activated extracellular signal-regulated kinase (Erk) in EDG-6 transfected cells in a pertussis toxin-sensitive manner. These results indicate that EDG-6 is a high affinity receptor for SPP, which couples to a $G_{i/o}$ protein, resulting in the activation of growth-related signaling pathways. (Blood. 2000;95:2624-2629)

Introduction

Sphingosine-1-phosphate (SPP) is a metabolite of complex sphingolipids that acts as both a second messenger and as a high-affinity ligand for cell surface receptors.[1] SPP is produced by sphingosine kinase that is activated in response to a variety of signals, including mitogens such as platelet-derived growth factor (PDGF) and serum,[2] G protein–coupled receptor agonists such as carbachol,[3] the cytokine TNF-α,[4] and ligation of Fc receptors.[5] Intracellular-formed SPP mediates release of Ca^{++} from intracellular stores,[3,5,6] stimulates several mitogenic and antiapoptotic signaling pathways,[7,8] and also contributes to the mitogenic response of fibroblasts to PDGF.[2,9] In further support of an intracellular mode of action for SPP, microinjection of SPP mobilizes calcium from internal sources,[3] is mitogenic for Swiss 3T3 fibroblasts,[9] and inhibits apoptosis of mouse oocytes induced by the antitumor drug doxorubicin.[10] Furthermore, overexpression of sphingosine kinase increases intracellular SPP and promotes cell growth and survival.[11]

However, because several responses to SPP are at least partially inhibited by pertussis toxin (PTX), which adenosine diphosphate (ADP) ribosylates and specifically inactivates $G_{i/o}$ proteins, and some require very low concentrations of SPP, it has been suggested that G protein–coupled cell surface receptors (GPCRs) might also be involved (reviewed in Speigel et al[12]). In agreement, a family of GPCRs, known as the endothelial differentiation gene (EDG) receptors, which specifically bind SPP or the related lipid, lysophosphatidic acid (LPA), has recently been identified.[13-18] The EDG family can be divided into 2 subfamilies based on amino acid sequence homology. The subfamily consisting of EDG-1, EDG-3, and EDG-5 display 40% to 45% sequence identity to each other and only 30% to 35% identity to the members of the other subfamily EDG-2 and EDG-4 (Figure 1). EDG-1, EDG-3, and EDG-5 have been shown to be SPP receptors,[13-16] whereas EDG-2, EDG-4, and EDG-7 are LPA receptors.[17-19]

Recently, a new member of the EDG family was cloned and named EDG-6.[20] EDG-6 is expressed in lymphoid and hematopoietic tissue as well as the lung.[20] Interestingly, EDG-6 does not clearly belong to either the SPP or the LPA subfamily of EDG receptors, as it displays a similar degree of homology to all 5 of the previously identified members. Thus, it was unclear whether EDG-6 is likely to be a receptor for SPP, LPA, or another related lysophospholipid. In this paper, we show that SPP binds specifically to EDG-6 and activates the mitogen-activated protein kinase (MAPK) signal transduction pathway.

Materials and methods

Materials

SPP, sphinganine-1-phosphate (dihydro-SPP), and sphingosylphosphorylcholine (SPC) were purchased from Biomol Research Laboratory Inc (Plymouth Meeting, PA). Lysophosphatidic acid was purchased from Avanti Polar Lipids (Birmingham, AL). γ-[^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Pertussis toxin (PTX) was from Research Biochemicals International (Natick, MA). Serum and medium were obtained from Biofluids (Rockville, MD). PathDetect Elk trans-Reporting System was from Stratagene (La Jolla, CA).

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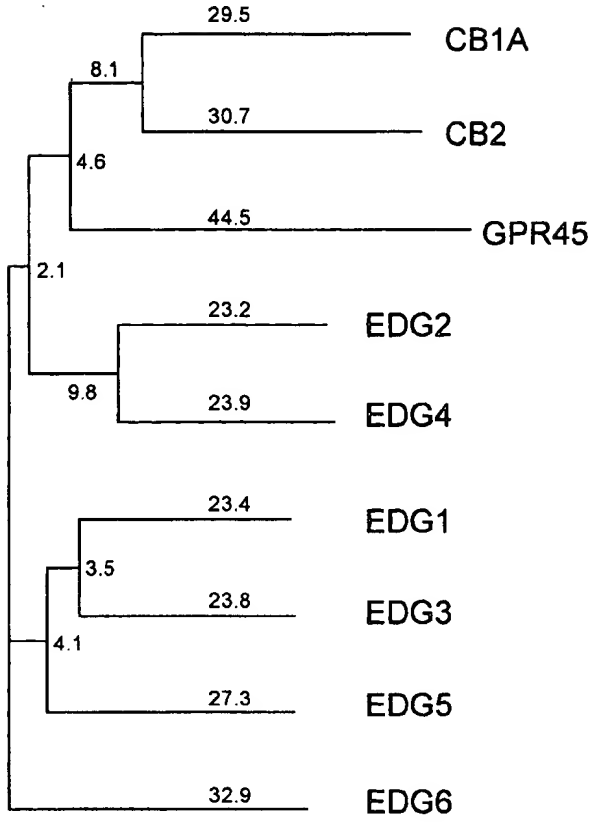


Figure 1. Phylogenetic tree of the human lysophospholipid-binding receptors, together with the next closest group of human cannabinoid receptors. GPR45 is the mammalian orthologue of the *Xenopus laevis* LPA receptor PSP24.[37] The numbers indicate the percentage of divergence. EDG-6 is more closely related to the SPP-binding receptors EDG-1, EDG-3, and EDG-5 than to the LPA-binding receptors EDG-2 and EDG-4.

Cell culture and transfection

Human embryonic kidney cells (HEK293, ATCC CRL-1573) and Chinese hamster ovary cells (CHO-K1) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. EDG-6 expression plasmids (RC/CMV containing c-terminal c-*myc*-tagged or N-terminal hemaglutinin [HA]-tagged EDG-6) were transfected into HEK293 or CHO-K1 cells using Lipofectamine Plus (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. The cells were then grown for 2 days to allow expression of receptors before the experiments were performed. In some experiments, cells were cotransfected with pCEFL GFP, which encodes green fluorescent protein. Transfection efficiencies were typically 30% to 35%. CHO-K1 cells stably transfected with c-*myc*-tagged human EDG-6 were grown in DMEM containing 10% fetal bovine serum and 0.4 g/L G418 sulfate (Biofluids).

Fluorescence-activated cell sorter (FACS) analysis

The human EDG-6 receptor was N-terminal tagged with an HA-epitope (peptide sequence: MGYPYDVPDYAGGP) and C-terminal tagged with a c-*myc*-epitope. Construction, expression, and flow cytometry analysis were performed as described.[21] The N-terminal HA-epitope tag was detected with a fluorescein isothiocyanate (FITC)-labeled anti-HA antibody.

SPP binding assay

[^{32}P]SPP was synthesized enzymatically using recombinant sphingosine kinase as previously described.[22] Transfected cells were incubated with the indicated concentration of [^{32}P]SPP in 200 μL binding buffer (20 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 15 mmol/L NaF, 2 mmol/L deoxypyridoxine, 0.2 mmol/L phenyl-methyl-sulfonyl-fluoride [PMSF], 1 μg/mL aprotinin and leupeptin) for 30 minutes at 4°C. Unlabeled lipid competitors were added as 4 mg/mL fatty acid–free bovine serum albumin (BSA) complexes. Cells were washed twice with ice cold binding buffer containing 0.4 mg/mL fatty acid–free BSA, resuspended in phosphate-buffered saline (PBS), and bound [^{32}P]SPP was quantitated by scintillation counting.[14]

Extracellular signal-regulated kinase activation

Cells were seeded in 60-mm plates and transfected the following day with EDG-6 expression plasmid and HA-tagged extracellular signal-regulated kinase (Erk)2 (at a 2:1 ratio of HA-Erk2 to EDG-6) with Lipofectamine Plus. After 2 days, cells were treated as indicated and lysed by the addition of 0.5 mL lysis buffer containing 25 mmol/L 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.4, 0.3 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS), 0.5 mmol/L dithiothreitol (DTT), 20 mmol/L β-glycerophosphate, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 mmol/L Na_3VO_4, 1 mmol/L PMSF, and 10 μg/mL leupeptin for 10 minutes on ice. Lysates were centrifuged 15 minutes at 4°C. Anti-HA (2 μg) (Santa Cruz Biotechnology) was then added to lysates (800 μg protein) and incubated 2 hours at 4°C with rocking. Protein A/G Sepharose beads (Santa Cruz Biotechnology) (20 μL) were added and the incubation continued for an additional hour. The beads were pelleted and washed 3 times in lysis buffer and twice in kinase buffer (12.5 mmol/L HEPES pH 7.4, 10 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 12.5 mmol/L β-glycerophosphate, 0.5 mmol/L NaF, 0.5 mmol/L Na_3VO_4). The kinase assay was initiated by resuspending the beads in 50 μL of kinase buffer containing 50 μmol/L adenosine triphosphate (ATP), 0.5 mg/mL myelin basic protein (MBP), and 5 000 dpm/pmol γ-[^{32}P]ATP, and incubating 20 minutes at 30°C. The reaction was stopped by the addition of 12 μL of 6 ×-concentrated Laemmli sample buffer and the samples were boiled 5 minutes, separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. Nitrocellulose membranes were stained with Ponceau S (Sigma) to visualize protein bands and then exposed to film for autoradiography. Radioactivity was measured in a scintillation counter after cutting the radioactive bands. In some experiments, for the determination of Erk 1/2 phosphorylation, 15 μg of clarified whole cell lysate were resolved by 10% SDS-PAGE, and Erk 1/2 phosphorylation was detected by protein immunoblotting with rabbit polyclonal phospho-specific MAPK IgG (1:1 000, Promega), followed by horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (1:10 000; Amersham Pharmacia Biotech) as a secondary antibody. Erk 1/2 phosphorylation was detected by ECL (Amersham Pharmacia Biotech). Nitrocellulose membranes were stripped and reprobed using rabbit polyclonal anti-Erk2 IgG (Santa Cruz Biotechnology) to confirm equal loading.

Elk1-dependent transcription of a luciferase reporter

For the detection of Elk1-dependent transcription, the PathDetect in vivo signal transduction pathway reporting system (Stratagene) and the Dual-Luciferase reporter assay system (Promega) were used. CHO-K1 cells were seeded in 6-well plates and cotransfected the following day with 50 ng of the fusion activator plasmid pFA-Elk (Stratagene), 500 ng of the firefly luciferase reporter vector pFR-Luc (Stratagene), 100 ng of the *Renilla* luciferase control reporter vector (Promega), and 50 ng of 1 of the plasmids, pcDNA3.1(+) or pcDNA3.1(+) containing the human EDG-6 receptor or the human EDG-1 receptor. After 30 to 40 hours, cells were washed with PBS and serum-free medium was added. Two to 3 hours later, the cells were stimulated with SPP and incubated for an additional 5 to 6 hours. The medium was removed and the cells were incubated in 300 μL passive lysis buffer (Promega) for 30 minutes at room temperature. Luminescence of 20 μL aliquots was measured with the Berthold Luminat LB 9507 for 10 seconds after injection of 50 μL each of luciferase assay buffer II and Stop & Glo buffer (Promega).

Results

Overexpression of EDG-6 in HEK293 cells

Figure 1 shows a phylogenetic tree depicting the relationship of EDG-6 to other EDG family members, as well as the next most closely related group of receptors, the cannabinoid receptors. The LPA-receptors EDG-2 and EDG-4, as well as the SPP-receptors EDG-1, EDG-3, and EDG-5, are clearly distinct from each other and form their own subgroups. Within these 2 EDG-subgroups, EDG-6 has a slightly higher homology to the first and the seventh transmembrane domains of the SPP subgroup than to the LPA subgroup. EDG-6 has a 44% overall identity to EDG-1, 46% to EDG-3, and 42% to EDG-5, and less homology to the LPA subgroup, 39% to EDG-4 and 37% to EDG-2.[20] These homologies suggest that EDG-6 could be an SPP receptor. To examine this possibility, we transfected HEK293 cells, which do not express EDG-6, as determined by RT-PCR (data not shown), and have no detectable binding sites for SPP,[9,14] with C-terminal c-*myc* epitope tagged or N-terminal HA-tagged human EDG-6 cDNA. Expression of EDG-6 protein on the cell surface was examined by flow cytometric analysis with anti–c-*myc* or anti-HA antibodies. FACS analysis showed that intact HEK293 cells transfected with EDG-6-*myc* were indistinguishable from untransfected cells (Figure 2A). However, when EDG-6-*myc*–transfected HEK293 cells were permeabilized, a distinct shift in the fluorescence was detected, indicating that permeabilization allowed access of the c-*myc* antibody to the C-terminus that is located at the cytoplasmic tail of EDG-6 (Figure 2B). HEK293 cells transfected with N-terminal HA-tagged EDG-6 showed a shifted fluorescence without permeabilization (Figure 2C), indicating cell surface expression of HA-tagged EDG-6, as the N-termini of G protein–coupled receptors are located extracellularly. Interestingly, this peak was also slightly enhanced by cell permeabilization (Figure 2D), suggesting that some HA-tagged EDG-6 may be expressed intracellularly.

Binding of SPP to EDG-6

Having established that human EDG-6 is expressed on the surface of transiently transfected HEK293 cells, it was of interest to determine whether SPP binds to EDG-6. In agreement with previous reports,[9,13,14] no specific SPP binding was detected in HEK293 cells transfected with the vector alone, whereas HEK293 cells transfected with c-*myc*–tagged human EDG-6 (HEK293–EDG-6) displayed dramatically increased binding of [^{32}P]SPP, which was competed by 1000-fold molar excess of either unlabeled SPP or dihydro-SPP to a level similar to that seen in untransfected cells (Figure 3). Neither SPC nor LPA effectively competed with [^{32}P]SPP for binding to HEK293–EDG-6 cells at 1000-fold excess (1 μmol/L). A computer curve fitting of binding isotherms indicated that SPP binding to EDG-6 was saturable and displayed moderately high affinity (K_D = 63 nmol/L) (Figure 4).

Because many studies indicate that SPC and LPA can also bind to SPP receptors, we examined whether SPC or LPA might bind to EDG-6 with very low affinity. Competition binding experiments were performed using up to 10 μmol/L unlabeled lipids. As expected, SPP competed for [^{32}P]SPP binding very effectively, with 50% of the total binding competing at 46 nmol/L unlabeled SPP (Figure 5), close to the measured K_D. Dihydro-SPP was less effective than SPP, competing 50% of the total binding at 210 nmol/L. SPC was able to compete for binding of [^{32}P]SPP, but only at very high concentrations in the micromolar range (Figure 5B). In contrast, the related lysophospholipid LPA did not compete for [^{32}P]SPP binding to HEK293–EDG-6 cells at any concentration tested.

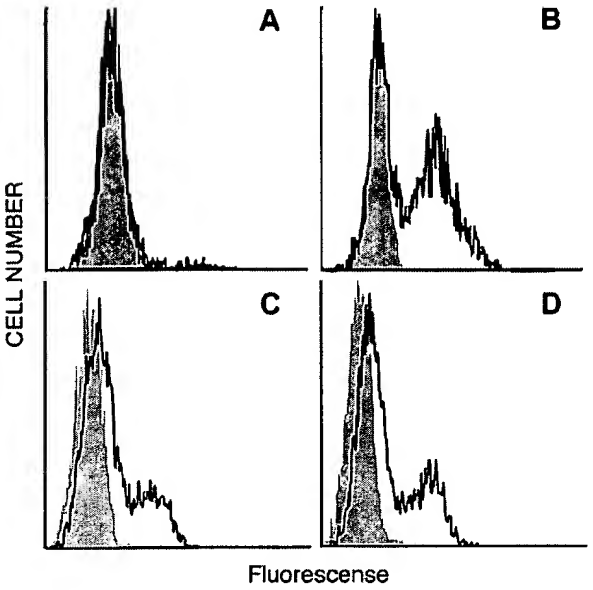


Figure 2. Expression of the N-terminal HA-tagged and the C-terminal *myc*-epitope–tagged human EDG-6 receptor. (A, C) Intact and (B, D) permeabilized HEK293 cells transfected with C-terminal *myc*-epitope–tagged human EDG-6 (A, B) or with N-terminal HA-tagged human EDG-6 (C, D). FACS analysis was performed as described in "Materials and methods." Grey areas indicate the signal without the first antibody (A, B) or autofluorescence of unstained cells (C, D), respectively.

Binding of SPP to EDG-6 activates Erk

The MAPK Erk is activated by a wide variety of G protein–coupled receptors,[23] including the known SPP receptors, EDG-1,[13,15] EDG-3,[24] and EDG-5.[25] To determine whether EDG-6 also activates Erk, HEK293 cells were cotransfected with EDG-6 and HA-tagged Erk2. SPP, markedly enhanced myelin basic protein phosphorylation in anti–HA-Erk2 immunoprecipitates from EDG-6 transfected HEK293 cells. However, Erk2 was also stimulated by SPP in vector-transfected cells, albeit to a lesser degree (data not

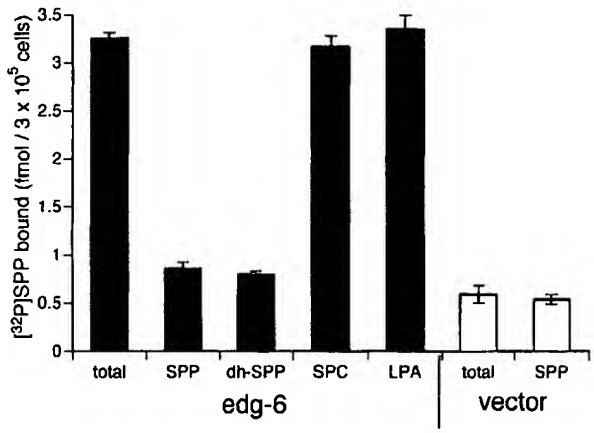


Figure 3. SPP binds specifically to HEK293 cells expressing EDG-6. HEK293 cells were transiently transfected with an expression plasmid containing the EDG-6 open reading frame (solid bars) or with vector alone (white bars); and binding of 0.5 nmol/L [^{32}P]SPP was measured in the absence (total) or presence of a 1000-fold excess of the indicated lipids as competitors as described in "Materials and methods." Results are means ± SD of triplicate determinations. Similar results were obtained in at least 3 independent experiments.

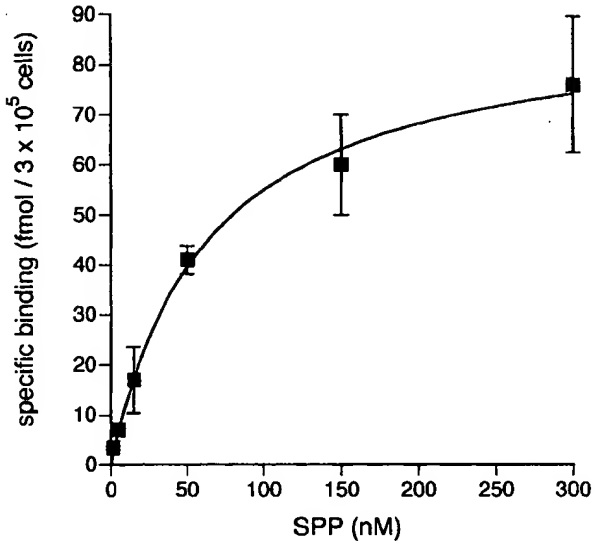
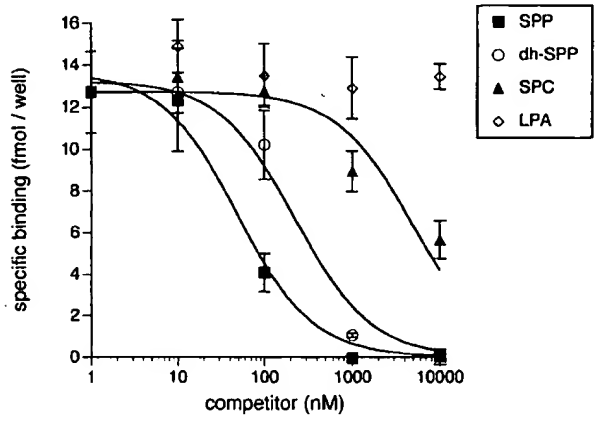


Figure 4. SPP binds to EDG-6 with high affinity. HEK293 cells were transfected with EDG-6 and specific binding of [^{32}P]SPP at the indicated concentrations was determined. "Specific binding" is binding in the absence of unlabeled SPP minus binding in the presence of excess unlabeled SPP. Results are means ± SD of triplicate determinations. The binding curve and the K_D (63 nmol/L) were calculated using Deltagraph for Macintosh.

shown). To circumvent the activation of Erk by SPP in untransfected HEK293 cells, which could be due to the presence of EDG-3 and EDG-5,[2,9,13,26] we used CHO-K1 cells, which only express low levels of EDG-5[24] and do not show Erk activation by SPP.[24,25] Moreover, CHO cells have previously been used successfully to examine Erk activation by the binding of SPP to cells overexpressing EDG-1, EDG-3, or EDG-5.[24,25,27] Expression of EDG-6 in CHO-K1 cells in the absence of SPP did not stimulate Erk, whereas SPP markedly activated HA-Erk2 in these cells, and as expected, SPP had only a slight effect on Erk2 activity in vector-transfected CHO-K1 cells (Figure 6A). Pretreatment with PTX completely eliminated SPP-stimulated Erk2 activity (Figure 6A). Moreover, in CHO-K1 cells stably expressing EDG-6, 100 nmol/L SPP activated endogenous Erk, as indicated by enhanced Erk phosphorylation (Figure 6C). SPP-stimulated Erk phosphorylation was completely PTX-sensitive. To examine whether Erk activation resulted in increased transcription, we used CHO-K1 cells and the PathDetect Elk trans-Reporting System, which detects Elk1-dependent transcription of a luciferase reporter. An advantage of this system is that the GAL4 DNA binding domain is a yeast transcriptional activator that is not recognized by mammalian transcription factors and thus yields very low backgrounds. Indeed, SPP did not enhance luciferase reporter activity in CHO cells transfected with empty pcDNA3.1 vector (Figure 6D), in agreement with the low endogenous expression of SPP EDG receptors in these cells. In contrast, SPP significantly increased luciferase activity after transfection with human EDG-1 or human EDG-6. Thus, similar to EDG-1,[13,15] EDG-6 also mediates SPP-induced MAPK activation, leading to phosphorylation and activation of Elk1.



competitor	Ki	correlation coefficient
SPP	46 nM	.988
dh-SPP	210 nM	.983
SPC	4.6 μM	.899

Figure 5. Specificity of lipid binding to EDG-6. HEK293 cells were transfected with EDG-6 and binding of 0.5 nmol/L [^{32}P]SPP was determined in the presence of the indicated concentrations of unlabeled lipid competitors. Results are means ± SD of triplicate determinations. Competition curves were fit and K_is calculated using Deltagraph program for Macintosh. Below, shown are the K_i values and correlation coefficients (r^2) of the curve fits for SPP, dihydro-SPP, and SPC. LPA did not compete at any concentration tested.

Discussion

EDG-6 displays high homology (37%-46% amino acid identity) to the previously identified members of the EDG family of G

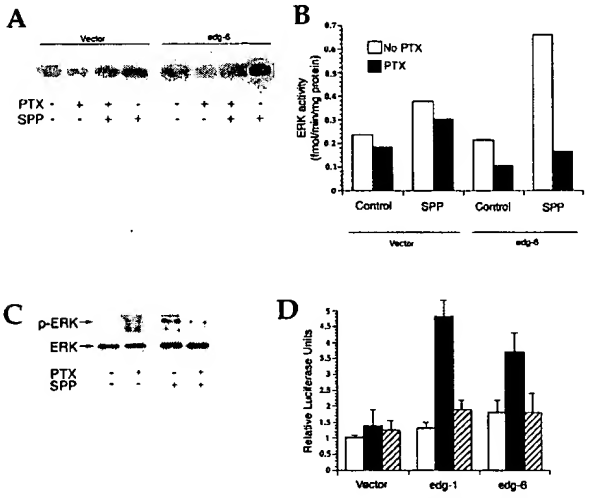


Figure 6. Activation of Erk by binding of SPP to EDG-6. (A) CHO-K1 cells were transiently cotransfected with an HA-tagged Erk2 and EDG-6 or an empty vector. Cells were treated without or with 200 ng/mL PTX for 3 hours, then stimulated with vehicle or 100 nmol/L SPP for 5 minutes. HA-Erk2 was immunoprecipitated from whole cell lysates and assayed for kinase activity using MBP as a substrate as described in "Materials and methods." Results are typical of 2 independent experiments. (B) ^{32}P incorporation into MBP was determined by scintillation counting. Data are expressed as picomole per minute per milligram and are the means of 2 separate experiments. White bars indicate the absence of PTX; solid bars, the presence of PTX. (C) CHO-K1 cells stably transfected with EDG-6 were treated without or with PTX for 3 hours, then stimulated with vehicle or 100 nmol/L SPP for 5 minutes, and Erk activation was determined by Western blot analysis with phospho-specific anti-Erk antibody. Blots were then stripped and reprobed with Erk antibody to determine total Erk levels. (D) Binding of SPP to EDG-6 stimulates Elk-1. CHO-K1 cells were cotransfected with the reporter plasmid pFR-Luc and the pcDNA vector alone or with either EDG-6 or EDG-1 inserts. Cells were treated without (white bars) or with 1 μmol/L SPP (solid and hatched bars) in the absence (solid bars) or presence of PTX (hatched bars) for 5 to 6 hours, and luciferase activity was measured as described in "Materials and methods." Data are means ± SEM.

protein–coupled receptors. Although by homology it cannot be clearly grouped with either the EDG-1, EDG-3, and EDG-5 subfamily, which bind SPP, or the EDG-2 and EDG-4 subfamily, which bind LPA, evidence presented here indicates that EDG-6 is an SPP receptor. SPP binds specifically to EDG-6 expressed on HEK293 cells, although with a lower affinity (63 nmol/L) than to EDG-1 (8 nmol/L),[13] EDG-3 (23 nmol/L), or EDG-5 (27 nmol/L).[14] Thus, residues conserved among EDG-1, EDG-3, and EDG-5 but not in EDG-6 may contribute to the increased affinity of SPP binding. Nevertheless, the affinity of EDG-6 for SPP is high enough to indicate that SPP could be a physiologically relevant ligand for EDG-6, as the concentration of SPP in plasma and serum is about 200 nmol/L and 500 nmol/L, respectively.[28]

Dihydro-SPP, which binds to EDG-1, EDG-3, and EDG-5 as potently as SPP, also binds to EDG-6, although with approximately a 5-fold lower affinity than SPP. Interestingly, SPC binds to EDG-6 with a 100-fold lower affinity than SPP (4.6 μmol/L). Similarly, SPC was recently shown to mobilize Ca^{++} in Xenopus oocytes expressing EDG-1, EDG-3, or EDG-5 at low micromolar concentrations, whereas SPP was active at nanomolar concentrations.[29] Moreover, SPC induced increased $[Ca^{++}]_i$ in HEL cells expressing EDG-1[27] or EDG-3,[24] and in K562 cells expressing EDG-5,[25] in each case with approximately a 100-fold lower efficacy than SPP. Thus, SPC may be a low affinity agonist for all 4 of the currently identified EDG SPP receptors. However, it is also possible that these effects were not mediated by SPC itself, as it was recently found that commercial preparations of SPC are contaminated with highly potent alkenyl-glycero-3-phosphates.[30] In contrast, SPC activated $I_{K(Ach)}$ in guinea pig atrial myocytes with EC_{50}s of 1.5 nmol/L.[31] Moreover, SPC activates signaling pathways different from those stimulated by SPP.[32,33] Thus, another sphingolipid receptor may exist that has a high affinity for SPC.

Binding of SPP to EDG-6 activates Erk, leading to the activation of the transcription factor Elk1. Activation of the MAPK Erk2 by EDG-6 in CHO-K1 cells was sensitive to PTX, suggesting that this response is mediated through a Gi/o protein. Similarly, EDG-1, EDG-3, and EDG-5 have all been shown to link to PTX-sensitive G proteins to mediate MAPK activation.[13,24,25] However, all 3 previously described EDG SPP receptors have also been shown to have PTX-insensitive effects as well, suggesting that EDG receptors can couple to multiple G proteins.

EDG-6 is expressed mainly in lymphocytes that would therefore be constantly exposed to high concentrations of SPP in serum. Thus, SPP signaling through EDG-6 may be maintained at a constant moderate level. The increase in release of SPP seen when platelets are activated may further enhance EDG-6 signaling. However, it is possible that EDG-6 signaling may be downregulated by constant exposure to SPP because it has previously been shown that ligand binding to GPCRs induces internalization of receptors[34] and SPP induces rapid trafficking of EDG-1.[13,26] It remains to be determined whether regulation of surface expression of EDG-6 is an important mechanism in determining how subsets of resting or activated lymphocytes respond to different SPP levels in the periphery or in tissue.

Although the biologic significance of SPP signaling through EDG-6 in lymphocytes and dendritic cells is poorly understood, the well-characterized growth-related or cytoskeleton-associated activities of SPP suggest that EDG-6 could be involved in various immunologic responses. Recently, it was shown that SPP protects human T-lymphoblastoma cells, which express EDG-3 and EDG-5, from apoptosis induced by Fas or ceramide.[35] Moreover, invasion of T-lymphoma cells into a fibroblast monolayer is dependent on SPP receptor-mediated RhoA and phospholipase C signaling pathways that lead to pseudopod formation and enhanced infiltration.[36] Thus, members of the EDG family may synergize with signaling pathways initiated by cytokines. Lysosphingophospholipids may play a critical role as potent autocrine and paracrine mediators in specific microenvironmental settings of normal and pathophysiologic immune responses.

Acknowledgment

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